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(54) Title: TREATING DISORDERS BY APPLICATION OF INSULIN-LIKE GROWTH FACTORS AND ANALOGS

(57) Abstract

A method of enhancing the survival of neuronal cells in a mammal, the cells being at risk of dying, the method comprising administering to the mammal an effective amount of at least one of the following substances: IGF-I; a functional derivative of IGF-I; IGF-II; or a functional derivative of IGF-II, provided that if IGF-I or IGF-II is administered, NGF or a functional derivative of NGF is also administered.

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TREATING DISORDERS BY APPLICATION OF
INSULIN-LIKE GROWTH FACTORS AND ANALOGS

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of Lewis et al. USSN 361, 595 filed on June 6, 1989.

The present invention relates to therapeutic polypeptides useful, e.g., for the treatment of neurological 5 and other disorders.

Insulin-like growth factors (IGFs) have been identified in various animal species as polypeptides that act to stimulate growth of cells in a variety of tissues (see Baxter et al., Comp. Biochem. Physiol. 91B:229-235 (1988); and 10 Daughaday et al., Endocrine Rev. 10:68-91 (1989) for reviews), particularly during development (see D'Ercole, J. Devel. Physiol. 9:481-495 (1987) for review). The IGFs, each of which has a molecular weight of about 7,500 daltons, are chemically related to human proinsulin: i.e. they possess A and B domains 15 that (1) are highly homologous to the corresponding domains of proinsulin, and (2) are connected by a smaller and unrelated C domain. A carboxyl-terminal extension, the D domain, is also present in IGFs but is not found in proinsulin.

Certain polypeptide fragments of the IGFs have proven 20 to be useful as antigens to raise antibodies specific for each of the IGFs (see, e.g., Japanese Patent Application No. 59065058; Hintz and Liu, J. Clin. Endocr. Metab. 54:442-446 (1982); Hintz et al., Horm. Metab. Res. 20:344-347 (1988)). Using labelled IGF-specific antibodies as a probe, IGF-I and 25 IGF-II (sometimes respectively termed "somatomedin C" and "somatomedin A") have been found in a variety of tissues, including the mammalian central nervous system(CNS); the presence in the CNS of mRNAs encoding these polypeptides

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suggests local synthesis in the CNS (see Baskin et al., TINS 11:107-111 (1988) for review). In addition, IGF-III (or "brain IGF"), a truncated form of IGF-I lacking the latter protein's three N-terminal amino acid residues, has been found in fetal
5   and adult human brain (Sara et al., Proc. Natl. Acad. Sci. USA 83:4904-4907 (1986), as well as in colostrum (Francis et al., Biochem. J. 251:95-103 (1988)). Two different IGF receptors have been identified in the adult human CNS (Baskin et al., 1988), including in the brain (Sara et al., Neurosci. Let.
10 34:39-44 (1982)). In addition, European Patent Application No. 86850417.6 describes evidence for a third type of IGF receptor located in human fetal membranes. Complicating research in this area are (1) evidence that the insulin receptor of brain membranes recognizes not only insulin but
15 also the IGFs; (2) the finding that one of the two types of adult IGF receptors exhibits some affinity for insulin as well as for both IGF-I and II, and (3) current uncertainty as to the physiological significance of binding of IGF-II to the second type of adult IGF receptor (Baskin et al., 1988).
20   IGF-I and IGF-II appear to exert a stimulatory effect on development or proliferation of a wide range of susceptible cell types (see Daughaday et al., 1989 for review). Treatment with the IGFs or with certain polypeptide fragments thereof has been variously suggested as a bone repair and replacement
25 therapy (European Patent Application No. 88303855.6), as a means to counteract certain harmful side effects of carcinostatic drugs(Japanese Patent Application No. 63196524), and as a way to increase lactation and meat production in cattle and other farm animals (Larsen et al., U.S. Patent No.
30 4,783,524). Each of the IGFs also appears to enhance the survival, proliferation and/or neurite outgrowth of cultured embryonic neurons (which, unlike mature neurons, have not yet lost their ability to undergo cell division) from various parts of the CNS (Aizenman et al., Brain Res. 406:32-42 (1987);

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Fellows et al., Soc. Neurosci. Abstr. 13:1615 (1987); Onifer
et al., Soc. Neurosci. Abstr. 13:1615 (1987); European Patent
Application No. 86850417.6, and from the peripheral nervous
system (Bothwell, J. Neurosci. Res. 8:225-231 (1982);

5   Recio-Pinto et al., J. Neurosci. 6:1211-1219 (1986)). In
addition, the IGFs have been shown to affect the development of
undifferentiated neural cells: human neuroblastoma tumor cells
were shown to respond to added IGFs by extending neurites
(Recio-Pinto and Ishii, J. Neurosci. Res. 19:312-320 (1988)) as

10   well as by undergoing mitosis (Mattson et al., J. Cell Biol.
102:1949-54 (1986). As the induction of the enzyme ornithine
decarboxylase has been shown to correlate with the stimulation
of mitotic activity of these cells, an assay for cell
proliferation has been developed based upon measuring the level

15   of activity of this enzyme (Mattsson et al., 1986).

       Developing forebrain cholinergic neurons (cultured rat
septal neurons) are sensitive to a variety of growth factors in
vitro. Addition of nerve growth factor (NGF) to the culture
medium increases the number of cells positive for the

20   expression of transmitter-specific enzymes (acetyl choline
esterase (AChE) and choline acetyl transferase (ChAT))
(Hartikka and Hefti, J. Neuroscience 8:2967-2985 (1988).
Thyroid hormone also increases the level of ChAT in cultured
septal neurons and thyroid hormone in combination with NGF

25   results in a stimulation of ChAT activity much greater than the
sum of the effects of individual addition of these two
substances (Hayashi and Patel, Dev. Brain Res. 36:109-120
(1987)). IGF-I, IGF-II, and insulin also induce ChAT activity
in cultured septal neurons (Knusel et al., J. of Neuroscience

30   10:558-570 (1990)). When NGF and insulin are both added to the
culture medium the effect on ChAT activity is additive, but the
effects of IGF-I or IGF-II in combination with insulin are not
additive (Knusel et al., 1990).

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In vivo studies also support the hypothesis that the
IGFs play a role in development and differentiation of the
immature peripheral and central nervous systems (Sara et al.,
J. Dev. Physiol. 1:343-350 (1979); Philipps et al., Pediatr.
Res. 23:298-305 (1988); Sara et al., Prog. Brain Res. 73:87-99
5   (1988)), although the physiological nature of this role remains
uncertain. Once the neuronal cells of the CNS reach maturity,
they do not undergo further cell division.

Neurotrophic factors other than the IGFs have been
proposed as a potential means of enhancing neuronal survival,
10 for example as a treatment for the neurodegenerative diseases
amyotrophic lateral sclerosis (using skeletal muscle-derived
proteins having apparent molecular weights in the~20,000-22,000
dalton and 16,000-18,000 dalton ranges: PCT Application No.
PCT/US88/01393), and Alzheimer's disease (using
15 phosphoethanolamine: PCT Application No. PCT/US88/01693).
Sara et al., although finding a "significant elevation" in
serum and cerebrospinal fluid somatomedin (IGF) levels in
patients suffering from Alzheimer's disease compared to normal
controls, nevertheless conclude:

20   Whether somatomedins play a casual (sic) role
                in the etiology of the dementia disorders of
                the Alzheimer type remains to be determined.
                However, since somatomedins stimulate the
                uptake of amino acids into brain tissue, their
                administration may provide beneficial
                therapeutic effects. Finally, the fall in
                somatomedins observed in normal elderly
25   patients raises the general question of their
                role in cell aging. (citation omitted; Sara et
                al., Neurobiol. Aging 3:117-120, 119 (1982)).

In a report that IGF-I, but not IGF-II, stimulates the
immediate (i.e. within 20 min.) release of acetylcholine from
30 slices of adult rat brain, a process thought to be related to
transitorily increased neurotransmission of acetylcholine
rather than to increased cholinergic enzyme activity, Nilsson
et al., Neurosci. Let. 88:221-226, 221, 224 (1988), point out
that

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> [One] of the major deficits in Alzheimer's
> disease concerns the cholinergic system of the
> brain, where a reduced synthesis and release of
> [acetylcholine] has been found....It is of
> considerable importance to further investigate
> the role of IGFs in neurodegenerative disorders
> such as Alzheimer's disease... (citations
> omitted).

Using antibody specific for IGF-I to detect an increase in the
presence of IGF-I in injured peripheral nerves, notably in the
non-neuronal cells named "Schwann cells", Hansson et al., Acta
Physiol. Scand. 132:35-41, 38, 40 (1988), suggest that

> Thus, increased IGF-I immunoreactivity is
> observed in regenerating peripheral nerves
> after any injury and seems to form part of a
> general reaction pattern, most evident in the
> Schwann cells. Our ultrastructural studies
> have revealed that the Schwann cells undergo
> hypertrophy after vibration trauma, and show
> signs of activation, i.e. the granular
> endoplasmic reticulum and Golgi complex
> increased in extent. We thus interpret the
> increase in IGF-I immunoreactivity in the
> Schwann cells, documented in this study on
> vibration-exposed nerves, as part of a
> transient, reactive response beneficial for the
> early stages of repair processes....We consider
> the increase in IGF-I immunoreactivity to
> reflect mainly the initial reactions in a chain
> of events resulting in repair of the injured
> tissue or organ [although this increase] may be
> interpreted to reflect disturbed axoplasmic
> transport [of IGF-I molecules], due in part to
> the diminution of microtubules reported to
> occur after vibration exposure. (citation
> omitted)

Further, Sjoberg et al., Brain Res. 485:102-108 (1989), have
found that local administration of IGF-I to an injured
peripheral nerve stimulates regeneration of the nerve as well
as proliferation of associated non-neuronal cells.

Several methods have been employed to decrease the
susceptibility of polypeptides to degradation by peptidases,
including, e.g., substitution of D-isomers for the
naturally-occurring L-amino acid residues in the polypeptide

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(Coy et al., Biochem. Biophys. Res. Commun. 73:632-8 (1976)).
Where the polypeptide is intended for use as a therapeutic for
disorders of the CNS, an additional problem must be addressed:
overcoming the so-called "blood-brain barrier," the brain
capillary wall structure that effectively screens out all but
selected categories of molecules present in the blood,
preventing their passage into the brain. While the blood-brain
barrier may be effectively bypassed by direct infusion of the
polypeptide into the brain, the search for a more practical
method has focused on enhancing transport of the polypeptide of
interest across the blood-brain barrier, such as by making the
polypeptide more lipophilic, by conjugating the polypeptide of
interest to a molecule which is naturally transported across
the barrier, or by reducing the overall length of the
polypeptide chain (Pardridge, Endocrine Reviews 7:314-330
(1986); U.S. Patent No. 4,801,575.

SUMMARY OF THE INVENTION

In general, the invention features a method of
enhancing the survival of neuronal cells at risk of death,
preferably non-mitotic neuronal cells and/or cholinergic
neuronal cells, in a mammal, preferably in the context of a
therapeutic treatment of neuronal tissues which are suffering
from the effects of aging, of injury, or of a disease e.g.,
Alzheimer's disease, stroke, epilepsy, amyotrophic lateral
sclerosis, or Parkinson's disease, by administering to the
mammal an effective amount at least one of the following:
IGF-I, a functional derivative of IGF-I, IGF-II, or a
functional derivative of IGF-II, with or without the
administration of an effective amount of NGF or a functional
derivative thereof, provided that, if IGF-I or IGF-II is
administered, NGF or a functional derivative thereof is
administered.

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The invention also features a method of enhancing the survival of neuronal cells at risk of death, preferably non-mitotic neuronal cells and/or cholinergic neuronal cells, in a mammal, preferably in the context of a therapeutic

5   treatment of neuronal tissues which are suffering from the effects of aging, of injury, or of a disease, e.g., Alzheimer's disease, stroke, epilepsy, amyotrophic lateral sclerosis, or Parkinson's disease, by treating said mammal with a first treatment including administration of a cell survival promoting

10   amount of a growth factor, e.g., IGF-1 or IGF-2, or a functional derivative of the growth factor (e.g., a fragment, analog, or analog of a fragment of the first growth factor), alone, or in a biologically active combination with another such growth factor or functional derivative, and then treating

15   said mammal with a second treatment including administration of a nerve transmitter increasing amount of a transmitter enhancer e.g., NGF, or a functional derivative of the transmitter enhancer (e.g., a fragment, analog, or analog of a fragment of the transmitter enhancer). In preferred embodiments fragments,

20   analogs, or analogs of fragments of IGF-I, IGF-II, or NGF are administered.

The invention also features a method of enhancing the cholinergic activity (i.e., acetylcholine-synthesizing capacity) of cholinergic neuronal cells in a mammmal,

25   preferably non-mitotic neuronal cells, and preferably in the context of a therapeutic treatment of neuronal tissues which are suffering from the effects of aging, of injury, or of a disease, e.g., Alzheimer's disease, stroke, epilepsy, amyotrophic lateral sclerosis, or Parkinson's disease, by

30   administering to the mammal an effective amount of one or more of the following: IGF-I, IGF-II, a functional derivative of IGF-I, or a functional derivative of IGF-II (preferably administering a fragment of IGF-I or IGF-II or, alternatively,

35   administering an analog of IGF-I, of IGF-II, or an analog of a

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fragment of IGF-I or IGF-II), with or without the
administration of an effective amount of NGF or a functional
derivative thereof, provided that if IGF-I or IGF-II is
administered, NGF or a functional derivative thereof is also
5   administered.

        The invention also features a method of enhancing the
cholinergic activity (i.e., acetylcholine-synthesizing
capacity) of cholinergic neuronal cells in a mammmal,
preferably non-mitotic neuronal cells, and preferably in the
10   context of a therapeutic treatment of neuronal tissues which
are suffering from the effects of aging, of injury, or of a
disease, e.g., Alzheimer's disease, stroke, epilepsy,
amyotrophic lateral sclerosis, or Parkinson's disease, by
treating said mammal with a first treatment including
15   administration of a cell survival promoting amount of a growth
factor, e.g., IGF-I or IGF-II, or a functional derivative of
the growth factor (e.g., a fragment, analog, or analog of a
fragment), along, or in a biologically active combination with
another such growth factor or functional derivative, and then
20   treating said mammal with a second treatment including an
administration of a nerve transmitter increasing amount of a
transmitter enhancer, e.g., a factor that increases the level
of a transmitter specific enzyme in the cell, e.g., NGF, or a
functional derivative of a transmitter enhancer (e.g., a
25   fragment, analog, or analog of a fragment).

        Another method of the invention features treating a
head or spinal cord injury of a mammal, or a disease condition
of a mammal, e.g., stroke, epilepsy, age-related neuronal loss,
amyotrophic lateral sclerosis, Alzheimer's desease, or
30   Parkinson's desease, by (1), administering to the mammal an
effective amount of at least one of the following substances:
IGF-I, a functional derivative of IGF-I, IGF-II, or a
functional derivative of IGF-II, with or without the
administration of NGF or a functional derivative thereof, or by

(2),treating said mammal with a first treatment including administration of a cell survival promoting amount of one or more of a first group of substances, e.g., IGF-I, a functional derivative of IGF-I, IGF-II, or a functional derivative of IGF-II, and then treating said mammal with a second treatment including administration of a nerve transmitter increasing amount of a transmitter enhancer or a functional derivative thereof, e.g., NGF or a functional derivative thereof.

The invention also features a method of modifying a ligand, preferably a neuroactive polypeptide, capable of binding to a receptor located on a cell surface, by first binding the ligand to a preparation of said receptor, then performing the modification procedure (preferably cationization, glycosylation, or increasing the lipophilicity of the polypeptide), and then releasing the modified ligand from the receptor.

Polypeptides administered in methods of the invention may be chemically modified in such a way as to increase the transport of the polypeptide across the blood-brain barrier, e.g., by modifications of the polypeptide that increase lipophilicity, alter glycosylation, or increase net positive charge.

Embodiments of the invention include the administration of more than one neuroactive polypeptide. In preferred embodiments the combined desired effect of administration of the polypeptides is additive, and in more preferred embodiments the effect is synergistic.

In other preferred embodiments, where a fragment of IGF-II is administered, IGF-II(54-67) is the preferred IGF-II fragment.

The invention also features a composition including a first component taken from the group of, purified IGF-I, a purified functional derivative of IGF I, purified IGF-II, or a purified functional derivative of IGF II, and a second

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component taken from the group of, purified NGF, or a purified functional derivative of NGF. Purified means that the substance is of 95% or greater (by weight) purity, i.e., that it is substantially free of proteins, lipids, and carbohydrates with which it is naturally associated.

The methods of the invention use IGF-I, IGF-II, functional derivatives of IGF-I and of IGF-II, combinations thereof, and combinations thereof which also include NGF or functional derivatives of NGF to enhance the survival rate and/or the cholinergic activity of mammalian cells at increased risk of death due to some factor such as disease, injury, or natural aging processes, or where stimulation of cholinergic activity could have a beneficial effect on the mammal's condition. Some of the functional derivatives utilized by the method of the invention are known; others may be discovered by applying the routine methods disclosed herein.

Methods (and compositions) of the invention, e.g., the joint administration of IGF-I and NGF, enhance the survival and neurotransmitter-synthesizing capacity of cholinergic neurons in a previously unknown, complimentary manner.

Survival of a treated neuronal cell denotes maintenance of the cell's viability to an extent greater than that of untreated control cells. Since the preponderance of neuronal cells of the mature CNS are commonly believed to be incapable of cell division, the ability of an agent to promote the survival of such cells maybe measured by an assay indicative of cellular trophic response, such as the ornithine decarboxylase assay disclosed herein. Alternatively, one can utilize any other assay which reproducibly indicates relative numbers of surviving cells, such as directly counting cells which stain as viable cells or which possess other characteristics of viable neurons, or assaying incorporation of appropriate labelled precursors into mRNA or protein. Where the effect of an added growth factor, functional derivatives,

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or a combination of growth factors and/or functional
derivatives on the functioning of cholinergic neurons is of
particular interest, an alternative assay which measures that
functioning, such as the choline acetyltransferase or acetyl
5   choline esterase assays disclosed herein, may be utilized.

     Any of these approaches may be adapted to test the
effect of treatment with growth factors, functional
derivatives, or combinations of growth factors and/or
functional derivatives on particular subsets of neurons known
10   to be vulnerable in specific degenerative diseases, such as
spinal cord cholinergic neurons in amyotrophic lateral
sclerosis. A preliminary screen for polypeptides which bind to
the IGF or NGF receptors may first be employed to indicate
likely candidates for the assays described above, e.g., the
15   cell survival or cholinergic activity assay; disclosed herein
is an IGF-I-receptor displacement assay designed for such a
purpose. Methods for measuring the ability of NGF or its
functional derivatives to bind its receptors are known to those
skilled in the art. Those polypeptides which appear to promote
20   cell survival or cholinergic activity under one or more of the
above assays may be further tested, by appropriate in vivo
administration, for their ability to counteract the
degenerative effects of aging, injury or disease in the nervous
system or other tissue of an animal.

25   The use of any polypeptide as a therapeutic raises the
issue of stability of the polypeptide after administration to
the organism, when it is exposed to the action of various
peptidases both within and without the target tissue. Where
lack of such stability is expected to be a problem, certain
30   stability-enhancing modifications disclosed herein may be made
to the polypeptide. Other modifications designed to facilitate
transport of the polypeptide across the blood-brain barrier may
be made to the polypeptide, as disclosed herein.

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The method of the invention is useful for therapeutically treating a disorder of a human or other mammal characterized by the death of cells, particularly neural cells, including disorders attributable to a disease or aging of, or
5   injury to, such neuronal cells. The neurotrophic peptides, including the IGFs and/or their functional derivatives, and combinations of IGFs and/or their functional derivatives with NGF or its functional derivatives are useful for the treatment of neurodegenerative diseases such as Alzheimer's disease,
10 stroke, epilepsy, amyotrophic lateral sclerosis and Parkinson's disease, as well as general age-related neuronal loss, conditions which have proven particularly intractable to treatment by alternative methods.

Other features and advantages of the invention will be
15 apparent from the following description of the preferred embodiments thereof, and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The drawings are first described.

Drawings

20 Fig. 1 is a graph illustrating the effect of IGF-I on the survival of cholinergic neurons in rat spinal cord cultures.

Fig. 2 is a graph showing the effect of IGF-II and IGF-III on the survival of cholinergic neurons in rat spinal
25 cord cultures.

Fig. 3 is a graph illustrating the effect of certain synthetic peptide fragments of IGF-I and IGF-II on the survival of cholinergic neurons in rat spinal cord cultures.

Fig. 4 is a graph depicting the effect on brain
30 ornithine decarboxylase activity of increasing doses of IGF-I injected into the brains of immature rats.

Fig. 5 is a graph showing the effect on brain ornithine decarboxylase activity of injection of IGF-I or synthetic peptide fragments of IGFs into the brains of immature
35 rats.

Fig. 6 is a graph depicting the effect on brain ornithine decarboxylase activity of injection of IGF-I into the brains of mature rats.

Fig. 7 is a graph illustrating the effect of an IGF-II derivative and of IGF-I on survival of cortical cells, as assessed by leucine incorporation.

Fig. 8 is a graph illustrating the effect of an IGF-II derivative and IGF-I on the survival of cortical neurons, as assessed by morphological characteristics.

Fig. 9 is a graph illustrating the additive effect of NGF (at saturating concentration) and IGF-I on ChAT activity in cultured rat septal cells.

Fig. 10 is a graph illustrating the additive effect of NCF and IGF (at saturating concentration) on ChAT activity in cultured rat septal cells.

Fig. 11 is a graph illustrating the effect of timed addition of NGF and IGF-I on ChAT activity in cultured septal cells.

Fig. 12 is a graph illustrating the effect of NGF and IGF-I on the number of AChE positive cells in septal cultures.

The Peptides

The present invention is directed, inter alia, to the modification of neuroactive polypeptides such as IGF-I and IGF-II and their functional derivatives, and their use, both with and without accompanying administration of NGF or functional derivatives of NGF, as therapeutics for certain neurological diseases or disturbances characterized by increased vulnerability of neurons to dying. A "neuroactive polypeptide" or "growth factor" is defined as a polypeptide which exerts a survival enhancing effect on neuronal cells: e.g., the IGFs, e.g., IGF-I and IGF-II, Nerve Growth Factor (NGF), Epidermal Growth Factor, Fibroblast Growth Factor, and insulin. A "functional derivative" of a polypeptide is a compound which is a fragment, an analog, or an analog of a

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fragment of that molecule and which possesses the desired
biological activity, herein defined as the ability to promote
survival and/or cholinergic activity of neuronal cells. A
"fragment" of a polypeptide refers to any polypeptide subset of
5   that polypeptide. An "analog" of a polypeptide refers to a
molecule having biological activity but possessing some
structural differences compared to the polypeptide: e.g., an
altered amino acid sequence, or the presence of additional
chemical moieties not normally a part of the molecule. Such
10   moieties (introduced, for example, by acylation, alkylation,
cationization, or glycosylation reactions) may improve the
molecule's solubility, absorption, transport, biological
half-life, etc. Alternatively, or in addition, some moieties
may decrease the toxicity of the molecule, or eliminate or
15   attenuate any undesirable side effect of the molecule.
Moieties capable of mediating such effects are disclosed in
Remington's Pharmaceutical Sciences (Mack Pub. Co., Easton, PA,
1980). Although some derivatives of IGF-I, IGF-II, and NGF may
be inoperative alone or in combination, a person skilled in the
20   art disclosed herein can recognize which are operative and
which are not, as will be explained in more detail below. A
"transmitter enhancer" is a polypeptide which causes an
increase in the level of a transmitter. NGF is an example of a
transmitter enhancer. A "transmitter" is a neurotransmitter,
25   e.g., acetyl choline. A "transmitter-specific enzyme" is an
enzyme present in neurons and involved in transmitter
metabolism, e.g., in the case of cholinergic neurons, acetyl
choline esterase (AChE) or choline acetyl transferase (ChAT).
A "neuronal cell" is a neuron.
30   Some of the compounds within the scope of this
invention are depicted in Table 1, which shows the amino acid
sequences (expressed using single-letter abbreviations as
defined in Table 2) of IGF-I, IGF-II, and a number of
functional derivatives of IGF-I and IGF-II. These derivatives

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were selected for study on the basis of one or more of the
following criteria, which are related to the ability to bind to
IGF-I or IGF-II receptors, and thus are useful for identifying
additional functional derivatives of the invention: (1)
5   conservation of amino acid sequence among species; (2) presence
of "conservative" amino acid substitutions among species (i.e.,
amino acids with similar shape, charge or other salient
characteristics); (3) receptor shielding of tyrosine residues
from radioiodination (Maly and Luthi, J. Biol. Chem.
10   263:7068-7072 (1988); (4) predominance of hydrophilic residues,
suggesting the location of a receptor-binding domain on the
surface of the polypeptide, a presumptive requirement for
receptor interaction; and (5) consideration of hydrophobic and
polar regions of three-dimensional models (e.g., Blundell et
15   al., Fed. Proc. 42:2592-2597 (1983)) and identifying therefrom
regions which are possible binding sites. Analagous factors
can be applied in the design of NGF functional derivatives.

        Since the ability of peptides to penetrate the
blood-brain barrier is related to their lipophilicity or their
20   net ionic charge, suitable modifications of these peptides
(e.g., by substituting pentafluorophenylalanine for
phenylalanine, or by conjugation to cationized albumin) to
increase their transportability (Kastin et al, Pharmac.
Biochem. Behav. 11:713-716 (1979); Rapoport et al., Science
25   207:84-86 (1980); Pardridge et al., Biochem. Biophys. Res.
Commun. 146:307-313 (1987); Riekkinen et al., Peptides
8:261-265 (1987)) may be important for their bioavailability
following administration outside the blood-brain barrier, and
these modifications are within the scope of the invention. In
30   addition, since bioavailability of peptides may be limited by
their susceptibility to degradation by proteases and peptidases
(Littlewood et al., Neurochem. Int. 12:383-389
(1988)),modifications of these peptides (e.g., replacement of

L-amino acids with D-amino acids) to increase their metabolic
stability (Coy et al., 1976) may also be important for their
therapeutic efficacy, and these modified peptides are also
within the scope of the invention.

5   Functional derivatives of the invention include, among
others, peptides which vary from the native IGF or NGF
molecules in any one or more of the following ways:

        1. Chemical modification of the amino and carboxyl
groups present at the respective ends of the peptides.

10   2. Replacement of one or more of the amino acid
residues in the native sequence with biologically compatible
other amino acid residues.

        3. Replacement of one or more of the amino acid
residues in the native sequence with chemically modified,
15 biologically compatible other amino acid residues.

        4. Deletion of one or more of the amino acid residues
in the native sequence.

        5. Repetition of one or preferably a sequence of
several amino acid residues in the native sequence, with or
20 without chemical modification to, or replacement or deletion
of, one or more of the members of the sequence.

        6. Cyclization, that is, joining the amino and
carboxyl ends of the linear peptide.

        7. Linkage of a IGF-I, IGF-II, NGF, or a functional
25 derivatives of any of IGF-I, IGF-II, or NGF with another
molecule such as a polypeptide (e.g., another fragment of
IGF-I, IGF-II, or NGF) or a carbohydrate, by means of a
disulfide, peptide, ester or other covalent bond.

        The invention also utilizes as a preferred subgroup
30 within the IGF functional derivatives described above, those
functional derivatives having the sequence:

R_1-AA_1-AA_2-AA_3-AA_4...AA_n-R_2, wherein AA_1, AA_2, AA_3,
AA_4...AA_n are amino acid residues of IGF or of the
IGF-peptide subsets or are conservative replacements for them

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as defined in Table 2, and n is any integer from 5 to 70 for
IGF-I functional derivatives and 5-67 for IGF-II functional
derivatives. R_1 is attached to the amino group of AA_1 and
selected from the group of hydrogen, lower (C_{1-6}) alkyl,

5   lower alkyl carbonyl, lower alkenyl, lower alkynyl, formyl,
lower (C_{6-10}) aryl, aroyl, aryloxy-carbonyl,
aralkyloxy-carbonyl, lower alkyloxycarbonyl, benzoyl, 1- or
2-thenoyl, nicotinoyl, dihydronicotinoyl,
N-alkyldihydronicotinoyl, isonicotinoyl, and

10   N-alkyldihydroisonicotinoyl. The carboxyl-terminal substituent
(R_2) of the peptides is selected from the following: OH;
NH_2; OR_3, wherein R_3 is a lower alkyl or a lower aryl;
OR_3OH, wherein R_3 is defined as above; and $NH-R_3$ or
$N(CH_3)R_3$, wherein R_3 is defined as above. Alternatively,

15   the carboxyl group of the carboxyl-terminal amino acid may be
replaced by any one of $-PO_3H_2$, $-B(OH)_2$, $-CH_2OH$, $-SO_3H$
or a 5-tetrazole group.

       The invention also utilizes as a preferred subgroup
within the NGF functional derivatives described above, those

20   functional derivatives having the sequence:
$R_1-AA_1-AA_2-AA_3-AA_4...AA_n-R_2$, wherein AA_1, AA_2, AA_3,
$AA_4...AA_n$ are amino acid residues of NGF or its functional
derivatives or are conservative replacements for them as
defined in Table 2, and n is an integer corresponding to the

25   number of amino acid residues in NGF or in a functional
derivative thereof. R_1 is attached to the amino group of
AA_1 and selected from the group of hydrogen, lower (C_{1-6})
alkyl, lower alkyl carbonyl, lower alkenyl, lower alkynyl,
formyl, lower (C_{6-10}) aryl, aroyl, aryloxy-carbonyl,

30   aralkyloxy-carbonyl, lower alkyloxycarbonyl, benzoyl, 1- or
2-thenoyl, nicotinoyl, dihydronicotinoyl,
N-alkyldihydronicotinoyl, isonicotinoyl, and
N-alkyldihydroisonicotinoyl. The carboxyl-terminal substituent
(R_2) of the peptides is selected from the following: OH;

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NH$_2$; OR$_3$, wherein R$_3$ is a lower alkyl or a lower aryl;
OR$_3$OH, wherein R$_3$ is defined as above; and NH-R$_3$ or
N(CH$_3$)R$_3$, wherein R$_3$ is defined as above. Alternatively,
the carboxyl group of the carboxyl-terminal amino acid may be

5   replaced by any one of -PO$_3$H$_2$, -B(OH)$_2$, -CH$_2$OH, -SO$_3$H
or a 5-tetrazole group.

The amino-terminal amino group and/or the lysine,
serine or threonine side chains occurring within the peptide
may optionally be acylated by formyl, acetyl, propionyl, and

10   similar lower alkylacyl residues or by aryl or heterocyclic
acyl residues such as benzoyl, thenoyl, nicotinoyl,
isonicotinoyl, n-alkylnicotinoyland their dihydro and
tetrahydro derivatives. Such modifications would-be expected
to enhance the blood-brain barrier permeability of the

15   therapeutic agent (Creveling et al., Experientia 25:26-27
(1969); Bodor et al., Science 214:1370-1372 (1981)).

In peptide sequences containing proline, glutamic
acid, or aspartic acid at the amino-terminus, the amino
terminal amino acid may optionally be replaced by

20   L-pyroglutamic acid.

The fragment polypeptides of IGF-I, IGF-II, and NGF
are subsets of the IGF-I, IGF-2, and NGF molecules
(respectively) containing fewer amino acid residues than the
native molecules. Preferred IGF sequences are of 5-40 residues

25   and most preferred are sequences of 6-25 residues. A portion
of the amino acids of the fragments may be substituted with
conservative replacements or deletions which improve the
chemical or biological stability of the product peptides or
improve their transport across the blood-brain barrier.

30   Preferably, no more than 30% and more preferably no_more than
20%, of the amino acid residues are replaced or deleted. A
listing of suitable conservative replacements is given in Table
2, along with a key to the single-letter abbreviations for the
common, naturally-occurring amino acid residues found in

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proteins. Certain other abbreviations used in Table 2 are
herein defined: by Nle is meant norleucine, by Aib is meant
aminoisobutyric acid, by AdaA is meant ß-adamantylalanine, by
AdaG is meant α-adamantylglycine, by homo-Arg is meant

5   L-homoarginine, by D-homo-Arg is meant D-homoarginine, by Acp
is meant ε-aminocaproic acid, by Chg is meant
L-α-cyclohexylglycine, and by allo-Thr is meant
L-allothreonine. Additionally, by Cha is meant
ß-cyclohexyl-alanine, by Me is meant methyl (CH_3), by Orn is

10 meant ornithine, by pyro-Glu is meant the pyroglutamyl group,
by Met(O) and D-Met(O) are meant the sulfoxides derived from L-
and D-methionine, respectively, by ß-Ala is meant ß-alanine, by
Acm is meant acetamidomethyl, by L-Dopa is meant -
3-(3,4-dihydroxyphenyl)-L-alanine, and by Bpa is meant

15 4-benzoyl-phenylalanine.

The symbolism and abbreviations used are otherwise
those recommended by the IUPAC-IUB Joint Commission on
Biochemical Nomenclature, "Nomenclature and Symbolism for Amino
Acids and Peptides, Recommendations 1983" J. Biol. Chem.

20 260:14-42 (1985). As is conventional, these same symbols are
used to define the corresponding residues of the amino acids
when they are linked into a peptide chain. Where the amino
acid residue has isomeric forms, it is the L-form of the amino
acid that is represented unless otherwise expressly indicated.

25 In accordance with conventional representation, the amino group
at the N-terminus of each peptide appears to the left and the
carboxyl group at the C-terminus to the right.

Besides the amino acid substitutions suggested above,
other methods of improving transport of the polypeptide across

30 the blood-brain barrier, such as chemical modification of the
polypeptide, may be employed. In any chemical modification
procedure, the polypeptide may first be attached to its
receptor in order to protect and maintain the receptor-binding
site structure during the chemical modification process, which

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can comprise, for example,cationization (according to the
method, for example, of Pardridge et al., 1987) or
glycosylation (according to the method of Schwartz et al.,
Arch. Biochem. Biophys. 181:542-549 (1977)).

5   Uses of the Peptides

As described more fully below, the present invention
provides novel uses of IGF-I and IGF-II and their functional
derivatives, and of IGF-I, IGF-II, and their functional
derivatives in combination with NGF and its functional

10   derivatives, as agents for the treatment of diseases or
disturbances characterized by an increased risk of cell death,
including in particular, neuronal cell death. The bioactivity
of each polypeptide (or combination of polypeptides) of the
invention may be conveniently assayed by a brain ornithine

15   decarboxylase assay, a spinal cord choline acetyl transferase
assay, a cultured septal cell assay, or a cultured cortical
cell assay, all of which are described in detail below.
Alternatively, the polypeptides may first be screened by a
receptor-growth factor displacement assay, e.g., the

20   receptor-IGF-I displacement assay described below, which
measures the polypeptide's ability to displace labelled IGF-I
bound to receptors in homogenized brain tissue. This assay has
been demonstrated to correlate with the polypeptide's
bioactivity as measured by the two enzymatic assays. As

25   described in the examples below, these assays disclose
previously unknown bioactivity of IGF-I, IGF-II, IGF-III and
some functional derivatives of these molecules both alone, and
in combination with NGF or functional derivatives of NGF.
Thus, the peptides of this invention should be useful for

30   administration to humans or other mammals who suffer from
neurological diseases or disturbances characterized by
increased risk of neuronal cell death, as described above.
These neurological diseases or disturbances include but are not
limited to: Alzheimer's disease, Parkinson's disease,

35   amyotrophic lateral sclerosis, stroke, and concussive or
penetrating injuries of the brain or spinal cord.

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The formulations of this invention are useful for
parenteral administration, for example, intravenous,
subcutaneous, intramuscular, intraorbital, ophthalmic,
intraventricular, intracranial, intracapsular, intraspinal,
5   intracisternal, intraperitoneal, topical, intranasal, aerosol,
scarification, and also for oral, buccal, rectal or vaginal
administration. The compositions can be formulated for
parenteral administration to humans or other mammals in
therapeutically effective amounts (e.g., amounts which
10   eliminate or reduce the patient's pathological condition) to
provide therapy for the neurological diseases described above.

The compounds provided herein can be formulated into
pharmaceutical compositions by admixture with pharmaceutically
acceptable nontoxic excipients and carriers. As noted above,
15   such compositions may be prepared for use as parenteral
administration, particularly in the form of liquid solutions or
suspensions; for oral administration, particularly in the form
of tablets or capsules; or intranasally, particularly in the
form of powders, nasal drops, or aerosols.

20   · The compositions may conveniently be administered in
unit dosage form and may be prepared by any of the methods well
known in the pharmaceutical art, for example, as described in
Remington's Pharmaceutical Sciences. Formulations for
parenteraladministration may contain as common excipients
25   sterile water or saline, polyalkylene glycols such as
polyethylene glycol, oils of vegetable origin, hydrogenated
naphthalenes and the like. In particular, biocompatible,
biodegradable lactide polymer, lactide/glycolide copolymer, or
polyoxyethylene-polyoxypropylene copolymers may be useful
30   excipients to control the release of the peptides. Other
potentially useful parenteral delivery systems for these
peptides include ethylene-vinyl acetate copolymer particles,
osmotic pumps, implantable infusion systems, and liposomes.
Formulations for inhalation administration contain as

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excipients, for example, lactose, or may be aqueous solutions
containing, for example, polyoxyethylene-9-lauryl ether,
glycocholate and deoxycholate, or oily solutions for
administration in the form of nasal drops, or as a gel to be
5   applied intranasally. Formulations for parenteral
administration may also include glycocholate for buccal
administration, methoxysalicylate for rectal administration, or
citric acid for vaginal administration.

        The materials of this invention can be employed as the
10   sole active agent in a pharmaceutical or can be used in
combination with other active ingredients, e.g., other growth
factors which could facilitate neuronal survival in
neurological diseases, or peptidase or protease inhibitors.

        The concentration of the compounds described herein in
15   a therapeutic composition will vary depending upon a number of
factors, including the dosage of the drug to be administered,
the chemical characteristics (e.g., hydrophobicity) of the
compounds employed, and the route of administration. In
general terms, the compounds of this invention may be provided
20   in an aqueous physiological buffer solution containing about
0.1 to 10% w/v compound for parenteral administration. Typical
dose ranges are from about 1 µg/kg to about 1 g/kg of body
weight per day; a preferred dose range is from about 0.01 mg/kg
to 100 mg/kg of body weight per day. The preferred dosage of
25   drug to be administered is likely to depend on such variables
as the type and extent of progression of the neurological
disease, the overall health status of the particular patient,
the relative biological efficacy of the compound selected, the
formulation of the compound excipients, and its route of
30   administration.

        The present invention will be further illustrated by
the following examples. These examples are not be construed as
limiting the scope of the invention, which is to be determined
solely by the appended claims.

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EXAMPLE 1

Recombinant human IGF-I, IGF-II, and IGF-III, as well as several chemically synthesized peptides consisting of partial sequences of IGF-I or IGF-II, were obtained from commercial sources as indicated in Table 1. ^{125}I-labeled
5   [Threonine^{59}]IGF-I was obtained from Amersham (Arlington Heights, IL). Other peptides consisting of partial sequences of IGF-I or IGF-II were chemically synthesized using Fmoc chemistry on a Milligen Biosearch Model 9600 Peptide Synthesizer, and purified on Hewlett-Packard Models 1050 and
10 1090M HPLCs according to the method of Hudson, J. Org. Chem.53:617-624 (1988). Fmoc amino acids, BOP (Castro's reagent), and resins were purchased from Biosearch (San Raphael, CA 94901) and Bachem Bioscience, Inc. (Philadelphia, PA 19104). Solvents were purchased from Burdick and Jackson
15 (Muskegon, MI 49442). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO 63178).

Brain tissue containing the cerebral cortex and cerebellum was dissected from adult Sprague-Dawley rats (Hilltop Lab Animals, Inc. Scottsdale, PA) and homogenized at
20 low power for 5 minutes in a Brinkmann Polytron homogenizer (Westbury, NY) containing 50 volumes of ice-cold buffer consisting of 10 mM HEPES, 0.5% BSA, 0.0125% NEM, 0.025% bacitracin, and 100 KIU/ml aprotinin, pH 7.6 (Bohannon et al., Endocrinology 119:943-945 (1986). Following homogenization,
25 the tissue was collected after centrifugation at 7800 x g for 20 minutes and resuspended in 10 volumes of assay buffer. Tissue (50 µl), 100 µl ^{125}I-[Threonine^{59}]IGF-I (20 pM), and 50 1 of buffer or peptides of varying concentration were added to 96-well plates and incubated on ice for 3 hours.
30 After the incubation period, the tissue was collected on Whatman GF/C filters that had been pre-soaked in 0.01% polyethylenimine and washed four times with ice-cold assay buffer using a Brandel cell harvester (Gaithersburg, MD). The filters were removed and the bound ^{125}I-[Threonine^{59}]IGF-I
35 was measured using a Beckman Model 5500B Gamma Counter.

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Table 3 summarizes the results of the ^{125}I-[Threonine^{59}]IGF-I displacement assay utilizing native IGFs and IGF fragments. The results demonstrate that, while IGF-I and IGF-III are potent displacers of ^{125}I-[Threonine^{59}]IGF-I, IGF-II is essentially inactive, indicating that the assay is selective for the identification of IGF-I-like molecules. In this assay, IGF-I(24-41) alone or in combination with IGF-II(54-67) were active in displacing ^{125}I-[Threonine^{59}]IGF-I. IGF-II(54-67) alone, and several other fragments listed in Table 3 were not significantly effective displacers of ^{125}I-[Threonine^{59}]IGF-I.

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EXAMPLE 2

Brains were removed intact from adult Sprague-Dawley
rats, frozen on powdered dry ice, and cut into 20μm sections
(at the level of the cerebellum and brain stem) which were
thaw-mounted onto gelatin-coated glass microscope slides
5   (Herkenham and Pert, J. Neurosci. 2:1129-1149 (1982)). Using a
modification of the method of Bohannon et al. (1986), the
tissue sections were covered with 250 μl of HEPES assay
buffer (see Example 1) containing 0.01 nM
^{125}I-[Threonine^{59}]IGF-I alone or in combination with
10   unlabeled IGF-I, IGF-II, or synthetic peptide fragments
thereof. The sections were incubated at 4°C for 24 hours and
then rinsed in three 1-minute changes (200 ml each) of ice-cold
HEPES assay buffer. The tissue sections were then wiped off
the slides with filter paper, and the tissue-bound
15   radioactivity was measured in a Beckman Model 5500B Gamma
Counter.

In this assay, in contrast to the assay described in
Example 1, ^{125}I-[Threonine^{59}]IGF-I binding was potently
displaced by both IGF-I and IGF-II, indicating the utility of
20   this assay for detecting potentially active derivatives of both
of these molecules (Table 4). ^{125}I-[Threonine^{59}]IGF-I
binding was displaced by IGF-II(33-40), but not by
IGF-II(54-67).

EXAMPLE 3

25   The activity of IGF-I, IGF-II, or synthetic peptide
derivatives of these molecules was assayed on dissociated
cultures of 14-day embryonic rat spinal cord neurons. The
spinal cord neurons were obtained from trypsin-dissociated
spinal cords, plated, incubated with peptides, and subsequently
30   (48 hr later) assayed for choline acetyltransferase activity as
described by McManaman et al., Dev. Biol. 125:311-320 (1988).

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In this assay, IGF-I was found to produce a substantial, dose-dependent increase in choline acetyltransferase activity (Fig. 1), suggesting that IGF-I can dramatically enhance the cholinergic activity of spinal cord
5 cholinergic neurons. Furthermore, IGF-II and IGF-III were found to be active in the spinal cord assay (Fig. 2). In addition, IGF-I(24-41) and IGF-II(33-40) were also found to produce a dose-dependent increase in choline acetyltransferase activity, indicating that each peptide is an active IGF
10 functional derivative (Fig. 3).

EXAMPLE 4

The _in vivo_ activity of IGF-I, IGF-II or synthetic peptide derivatives of these molecules was tested using a biochemical marker for CNS neurotrophic activity, the induction
15 of brain ornithine decarboxylase. The induction (i.e. increased activity) of ornithine decarboxylase has been reported to be a general marker for the actions of a variety of trophic factors. (Schwartz et al., Dev. Brain Res. 1:403-413 (1981); Kanje et al., Brain Res. 381:24-28 (1986); Russell et
20 al., Life Sci. 19:1297-1306 (1976);MacDonnell et al. Proc. Natl. Acad. Sci. USA 74, 4681-4684 (1977); Rinehart et al. Proc. Natl. Acad. Sci. USA 82, 4365-4368 (1985)).

Sprague-Dawley rats, 4 days old, were injected intracerebrally (in the area of the lateral ventricle) with
25 5 μl of 0.1 M phosphate-buffered saline (PBS) containing IGF-I, IGF-II or a synthetic peptide derivative (1.25-2.5 μg dose, with 6 animals per treatment group). After 6 hours, the brains were removed, and ornithine decarboxylase was assayed essentially as described by Lewis et al., Proc. Natl. Acad.
30 Sci. USA 75:1021-1023 (1978).

Administration of IGF-I produced a dose-dependent increase in brain ornithine decarboxylase activity (Fig. 4). In addition, both IGF-I(24-41) and IGF-II(54-67) increased brain ornithine decarboxylase activity (Fig. 5; these peptides
35 are referred to in Fig. 5 as IGF-I (2-4) and IGF-I(5-6), respectively).

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EXAMPLE 5

To determine whether the induction of brain ornithine decarboxylase by IGF-I was limited to developing animals, IGF-I was also injected intraventricularly into the lateral ventricles of adult Sprague-Dawley rats. After 6 hours, the
5   brains were removed, dissected into several regions (cerebral cortex, medial septum, and hippocampus), and then assayed for ornithine decarboxylase activity as described in Example 4. As shown in Fig. 6, IGF-I stimulated ornithine decarboxylase activity in all brain regions assayed. This result indicates
10   that IGF-related molecules have potential utility in widespread regions of the brain.

EXAMPLE 6

The ability of IGF-I and a synthetic derivative of IGF-II (IGF-II(54-67)) to increase the incorporation of $[^3H]$-leucine and to promote the survival of neurite bearing cells was examined in cultured rat cortical cells (the numbers "54-67" in IGF-II indicate the fragment includes amino acid residues 54-67 of native IGF-II). IGF-II(54-67), like IGF-I, increased $[^3H]$-leucine incorporation in low density 24 hour
20   mixed cortical cultures, as shown in Fig. 7. IGF-II(54-67) also displayed IGF-I-like survival-promoting activity in that it increased the survival of cortical neurons (as determined by the presence of neurite bearing cells), as shown in Fig. 8.

Measurements were performed, using standard techniques
25   known to those skilled in the art, on dissociated cortical cells obtained from day 18-19 embryonic rats. Cells were seeded at 1.5 x 10^4/cm^2 on poly-1-ornithine-laminin coated plastic tissue culture cells in serum-free N2 medium (Bottenstein et al. Proc. Natl. Acad. Sci. U.S.A. 76: 514-517
30   (1978)). $[^3H]$-leucine was added to cells at plating for the incorporation assay. Cultures were terminated 24 hours after plating and measured for either $[^3H]$-leucine incorporation of for the number of neuritic cells by microscopic examination.

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EXAMPLE 7

The effect of simultaneous administration of IGF-I and NGF on ChAT activity was assayed in cultured septal neurons. ChAT is the initial enzyme in the synthesis of the neurotransmitter, acetylcholine, and is a specific biochemical marker for cholinergic neurons. Assay of this enzyme may be used as an indication of the effects of IGF (and other factors) on the survival of cholinergic neurons and/or regulation of this enzyme. An additive increase in ChAT activity was seen with saturating concentrations of NGF combined with saturating or sub-maximal concentrations of IGF-I, as shown in Fig. 9. In Fig. 9 open squares represent IGF-I, diamonds indicate IGF-I + 2nM NGF, open circles indicate 2nM NGF, and the horizontal line at 403 DPM represents uninduced cells. A similar additive effect was seen when saturating concentrations of IGF-I were combined with saturating or sub-maximal concentrations of NGF, as shown in Fig. 10. In Fig. 10 open squares indicate NGF, diamonds indicate NGF + 25nM IGF-I, open circles represent 25nM IGF-I, and the horizontal line at 554 DPM represents uninduced cells. The percent increases in ChAT activity over control uninduced cells are summarized in Table 5.

Cultured rat septal cell experiments were performed generally as described in Hartikku and Hefti, J. Neuroscience, 8:2967-2985 (1985), Hayashi and Patel, Dev. Brain Res., 36:109-120 (1987), and as follows. Dissociated cell cultures of the septal region of day 17 embryonic rats were prepared by standard techniques known to those skilled in the art, using enzymatic (Dispase, Collaborative Research) dissociation of tissue. Cells were seeded (plated) at 6×10^5 cells/cm^2 in poly-1-ornithine-laminin coated plastic tissue culture wells, and cultured in serum-free N2 medium (Bottenstein et al., 1978) for 5 days without feeding. Control (uninduced) cultures received no added growth factors; induced cultures received the concentrations of IGF-I and NGF indicated in Figs. 9 and 10 at

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the time of plating. NGF is commercially available. ChAT was
assayed by the method described in McManaman, et al. Dev. Biol.
125:311-320 (1988). AChE staining was performed according to
the method of Hartikka and Hefti, J. Neuroscience 8:2967-2985
5   (1988).

Positive cyotchemical staining for the enzyme
acetylcholinesterase (AChE) has been shown to be a reliable
marker for choline acetyltransferase positive neurons in rat
septal cell cultures (Hartikka and Hefti, J. Neuroscience
10   8:2967-2985 (1988).

EXAMPLE 8

The sequence in which NGF and IGF-I are added to the
culture medium has a significant effect on the magnitude of the
increase of ChAT activity in cultured rat septal cells, as
15   shown in Fig. 11. In Fig. 11 A represents 2nM IGF, B
represents 25nM IGF-I, C represents IGF-I + NGF, both added 5
days before assay, and D IGF-I added at the beginning of the
experiment + NGF added on day 3 of the experiment, with assay
on day 5 of the experiment. When added separately, NGF or
20   IGF-I increased ChAT activity 50 to 60% in a 5 day old
culture. When NGF and IGF-I were present together for the
entire 5 days the NGF and IGF-I effects on ChAT activity were
additive (a 100% increase), as shown in Figs. 9, 10, and 11.

When IGF-I was present from the beginning of the
25   experiment and NGF was added on day 3, the ChAT activity on day
5 was increased by 300% over uninduced cultures, as shown in
Fig. 11. Thus it has been discovered that IGF-I and NGF act in
a previously unknown, complimentary manner to enhance the
survival and neurotransmitter-synthesizing capacity of -
30   cholinergic neurons.

Cultured rat septal cell experiments were performed as
described above.

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EXAMPLE 9

We have shown that under specific culture conditions
(4×10^5 cells/cm^2 in the presence of medium containing 10%
bovine calf serum), IGF-I increased the number of AChE positive
cells by 3-4 fold over control, growth factor-free cultures,
Fig. 12. In Fig. 12 A represents uninduced cells, B represents
cells treated with 2nM NGF, C represents cells treated with
100nM IGF-I, and D represents cells treated with NGF + IGF-I.
(DPM= disintigrations per minute.) NGF under the same
conditions, did not affect the number of AChE positive cells.
These results indicate that IGF-I has a greater effect on
cholinergic cell survival (i.e. increases cholinergic
survival), while NGF regulates (increases) ChAT activity in
existing cholinergic neurons.

EXAMPLE 10

Cationization is a process by which free carboxyl
groups of acidic amino acid residues on a polypeptide (i.e.,
aspartic acid and glutamic acid residues) are modified in order
to increase the net positive charge on the polypeptide. The
process of cationization has been used to enhance the cellular
uptake of large molecules such as albumin and horseradish
peroxidase into mouse fibroblast cells (Shen et al., Proc. Nat.
Acad. Sci. USA 75:1872-1876 (1978)). Kumagai et al., J. Biol.
Chem. 262:15214-15219 (1987), using intact microvessels from
bovine brain that are reportedly a model system for measuring
transport across the blood-brain barrier, showed that uptake of
cationized albumin by isolated bovine brain microvessels was
enhanced when compared with uptake of native albumin.

For global modification of free carboxyl groups, the
polypeptide (e.g., NGF, IGF-I, IGF-II or a functional
derivative) would be reacted with excess hexamethylenediamine
(HMD) (15.5 g/g total protein) for 30 minutes at room
temperature, followed by covalent coupling of HMD with
1-ethyl-3[-3-dimethyl- aminopropyl] carbodiimide hydrochloride

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(EDAC) (1.0g/g total protein) for 3 hours at room temperature. Unreacted species may be removed by filtration using Centricon-3 MPS-1 separation devices (Amicon, Danvers, MA) or ion exchange chromatography. The purified polypeptide may be analyzed using isoelectric focusing to determine the amount of cationization.

If the global modification is used on a polypeptide that is a ligand which binds to a cell surface receptor, and the modification produces a molecule lacking biological activity, the cationization process may be repeated as described above except that the polypeptide would be pre-bound to an appropriate receptor prior to cationization, in order to protect the receptor-binding site on the polypeptide. This protection procedure would be carried out as follows: Tissue, e.g., brain, containing receptors for the polypeptide of interest (e.g., IGF-I) is prepared as described above in Example 1. After incubation with the polypeptide ligand for 2 hours @ 4°C to permit receptor binding, the reaction mixture is brought to room temperature, and the cationization procedure carried out using HMD and EDAC as described above. The reaction mixture is then centrifuged at 16,000 rpm at 4@C for 30 sec in an SS-34 rotor in a Sorvall RC5B centrifuge. The supernatant is discarded and the pellet washed three times in PBS with bovine serum albumin (1 mg/ml). The pellet is resuspended in 100mM acetic acid and incubated for 10 min @ 4°C to release the cationized polypeptide from its receptors. After centrifugation again at 16,000 rpm, the supernatant, which contains the released cationized polypeptide, is pH-neutralized with NaOH. It may then be analyzed by isoelectric focusing, by a receptor-binding assay as described in Example 1, or by any appropriate assay for biological activity.

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EXAMPLE 11

An alternative to the global modification method is to couple polylysine to at least one free carboxyl group on polypeptide (such as IGF-I, IGF-II, or a functional derivative of either) with or without receptor protection as described
5   above in Example 10. The procedure follows the method of Shen et al., 1978. For example, polylysine, IGF-I and carbodiimide are added in a 1:1:1 ratio in water or buffer for 3 hours at room temperature. The modified protein would be separated and analyzed as described above in Example 10.

10   EXAMPLE 12

A third method for modifying protein carboxyl groups to enhance blood brain barrier transport is to form esters with diazomethane or N,N-dimethylformamide R acetals (DMF acetals), where R is dimethyl, diethyl, dibutyl, dibenzyl, etc. This
15   type of modification rapidly forms esters from negatively charged carboxylic acid groups, thus increasing the overall positive charge. An additional benefit from this modification is that these added ester groups may be such that they increase the overall lipophilicity of the polypeptide and may be removed
20   by intrinsic esterases in vivo to yield intact growth factor. The procedure for this modification, with or without receptor protection as described above in Example 10, is to react diazomethane or DMF acetals with the polypeptide in a 1:1 ratio in solution for 30 min. at room temperature, followed by
25   purification and characterization as described above in Example 10.

EXAMPLE 13

A fourth method of cationization, with or without receptor protection as described above in Example 10, combines
30   the advantages of polylysine cationization with the formation of cleavable esters to enhance blood-brain barrier transport, as well as to yield intact growth factor following transport. Polylysine may be made reactive by reaction with

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benzyloxylacetyl chloride followed byhydrogenation and mild
esterification procedures (Hassner et al., Tet. Let.
46:4475-4478 (1978); Mihara et al., Int. J. Peptide Protein
Res. 28:141-145 (1986)). Alternatively, DMF acetal derivatives
5   capable of reacting with polylysine could be used to link
polylysine to free carboxyl groups using ester linkages.

        EXAMPLE 14
        A further type of polypeptide modification is
10 glycosylation: the introduction of glucose or similar residues
by reductive amination using, for example, glucose and sodium
cyanoborohydride ($NaCNBH_3$). Glycosylation of proteins has
been shown to enhance the cellular uptake of these proteins and
may prove useful for improving blood-brain barrier transport
15 (Smith et al., Pharm. Res., in press). The procedure for
glycosylation, with or without receptor protection as described
above in Example 10, is based on the method of Schwartz et al.,
1977, wherein a polypeptide such as IGF-I, IGF-II, or a
functional derivative of either is combined with glucose and
20 $NaCNBH_3$ in a molar ratio of 1:300:1600 in 200 mM phosphate
buffer at pH 7 for at least 24 hr at 37 C°. Unreacted entities
may be removed as described in Example 10, or with lectin
affinity chromatography. In previous studies using
glycosylated albumin, the modified albumin was taken up by rat
25 epididymal microvessels at a greater rate than was native
albumin (Williams et al., Proc. Nat. Acad. Sci. USA
78:2393-2397 (1981)).
        EXAMPLE 15
        Blood-Brain Barrier Transport Model: Method of Audus
30 et al., Ann.N.Y.Acad.Sci. 507:9-18 (1987).
        Microvessel endothelial cells are isolated from the
cerebral gray matter of fresh bovine brains. Brains are
obtained from a local slaughter house and transported to the
laboratory in ice cold minimum essential medium (MEM) with

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antibiotics. Under sterile conditions the large surface blood vessels and meninges are removed. The cortical gray matter is removed by aspiration, then minced into < 1 mm cubes. The minced gray matter is then incubated with 0.5% dispase (BMB,

5   Indianapolis, IN) for 3 hours at 37°C in a shaking water bath. Following the 3 hour digestion, the mixture is concentrated by centrifugation (1000 xg for 10 min.), then resuspended in 13% dextran and centrifuged for 10 min. at 5800 xg. Supernatant fat, cell debris and myelin are discarded and the crude

10   microvessel pellet is resuspended in 1mg/ml collagenase/dispase and incubated in a shaking water bath for 5 hours at 37°C. After the 5-hour digestion, the microvessel suspension is applied to a pre-established 50% Percoll gradient and centrifuged for 10 min at 1000 xg. The band containing

15   purified endothelial cells (second band from the top of the gradient) is removed and washed two times with culture medium (50% MEM/50% F-12 nutrient mix). The cells are frozen (-80°C) in medium containing 20% DMSO and 10% horse serum for later use.

         After isolation, approximately 5 X 10^5 cells/cm^2

20   are plated on culture dishes or 5-12 em pore size polycarbonate filters that are coated with rat collagen and fibronectin. 10-12 days after seeding the cells, cell monolayers are inspected for confluency by microscopy.

         Characterization of the morphological, histochemical

25   and biochemical properties of these cells has shown that these cells possess many of the salient features of the blood-brain barrier. These features include: tight intercellular junctions, lack of membrane fenestrations, low levels of pinocytotic activity, and the presence of gamma-glutamyl

30   transpeptidase, alkaline phosphatase, and Factor VIII antigen activities.

         The cultured cells can be used in a wide variety of experiments where a model for polarized binding or transport is required. By plating the cells in multi-well plates, receptor

35   and non-receptor binding of both large and small molecules can

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be conducted. In order to conduct transendothelial cell flux measurements, the cells are grown on porous polycarbonate membrane filters (Nucleopore, Pleasanton, CA). Large pore size filters (5-12 μm) are used to avoid the possibility of the
5   filter's becoming the rate-limiting barrier to molecular flux. The use of these large-pore filters does not permit cell growth under the filter and allows visual inspection of the cell monolayer.

        Once the cells reach confluency, they are placed in a
10 side-by-side diffusion cell apparatus (Crown Glass, Sommerville, NJ). For flux measurements, the donor chamber of the diffusion cell is pulsed with a test substance, then at various times following the pulse, an aliquot is removed from the receiver chamber for analysis. Radioactive or
15 fluorescently-labelled substances permit reliable quantitation of molecular flux. Monolayer integrity is simultaneously measured by the addition of a non-transportable testsubstance such as sucrose or inulin and replicates of at least 4 determinations are measured in order to ensure statistical
20 significance.

TABLE 1

IGF PEPTIDE SEQUENCES

Peptide Name	Sequence	Source	Cat #
Human IGF-I (Somatomedin-C)	GPETL CGAEL VDALQ FVCGD RGFYF NKPTG YGSSS- -RRAPQ TGIVD ECCFR SCDLR RLEHY CAPLK PAKSA	AMGEN[1]	14010
Human IGF-I (Somatomedin-C)	GPETL CGAEL VDALQ FVCGD RGFYF NKPTG YGSSS- -RRAPQ TGIVD ECCFR SCDLR RLEHY CAPLK PAKSA	PENINSULA[2]	9010 Lot 18378
IGF-I(4-70) (Human Brain IGF)	TLCGAEL VDALQ FVCGD RGFYF NKPTG YGSSS- -RRAPQ TGIVD ECCFR SCDLR RLEHY CAPLK PAKSA	KABIGEN[3] KABIGEN[3]	Lot 88:101G Lot S:28
IGF-I(24-41)	YFNKP TGYGS SSRRA PQT	PENINSULA[2]	7308 Lot 007942
	YFNKP TGYGS SSRRA PQT	BACHEM[4]	PGRO 080 Lot F297
	YFNKP TGYGS SSRRA PQT	Synthetic[5]	
IGF-I(30-41)	GYGSS SRRAP QT	PENINSULA[2]	7306 Lot 002251
IFG-I(62-70)	APLKP AKSA	PENINSULA[2]	7318 Lot 015726
IGF-I(24-32)	YFNKP TGYG	Synthetic[5]	
IGF-I(24-41)-AMIDE	YFNKP TGYGS SSRRA PQT-NH_2	Synthetic[6]	
IGF-I(33-41)-AMIDE	SSSRR APQT-NH_2	Synthetic[6]	
48-Acm-IGF-I(42-57)- AMIDE	Acm GIVDE CCFRS CDLRR L-NH_2	Synthetic[7]	

1724R

Peptide Name	Sequence	Source	Cat. #
IGF-I(33-41)	SSSRR APQT	Synthetic[5]	
IGF-I(28-41)	PTGYG SSSRR APQT	Synthetic[5]	
IGF-I(27-36)	KPTGY GSSSR	Synthetic[5]	
IGF-II(54-67)	ALLET YCATP AKSE	PENINSULA[2][8]	7308 Lot 010718
IGF-II(62-67)	TPAKS E		
IGF-II(33-40)	SRVSR RSR	PENINSULA[2]	7304 Lot 016905
IGF-II Somatomedin-A	AYRPS ETLCG GELVD TLQFV CGDRG FYFSR PASRV SRRSR GIVEE CCFRS CDLAL LETYC ATPAK SE	COLLABORATIVE[9] COLLABORATIVE[9]	LOT 89-0172 LOT 89-0401

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1. Amgen, Thousand Oaks, CA 91320

2. Peninsula Laboratories, Belmont, CA 94002

3. Kabigen AB, S-112 87, Stockholm, Sweden

4. Bachem, Inc., Torrance, CA 90505

5. Synthesized on a Biosearch Solid Phase Peptide Synthesizer Model 9600 using Fmoc-Amino Acids linked to p-Alkoxybenzyl Alcohol Resins supplied by Bachem Bioscience, Inc. Philadelphia, PA 19104.

6. Synthesized on a Biosearch Solid Phase Peptide Synthesizer Model 9600 using 4-(2',4'-Dimethoxyphenyl-fmoc-aminomethyl)-phenoxy Resin (AØ4719) supplied by Novabiochem, AG Laufelfingen, Switzerland.

7. Synthesized on a Biosearch Solid Phase Peptide Synthesizer Model 9600 using the resin identified in footnote 6. Acm = Acetamidomethyl substituent on the cysteine side-chain sulfur atom.

8. This compound is incorrectly listed in the Peninsula Laboratories catalog as "Insulin-like Growth Factor 1 (57-70)".

9. Collaborative Research, Inc., Bedford, MA 01730

TABLE 2

CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid

	Code	Replace with
Alanine	A	D-Ala, Gly, Aib, B-Ala, Acp, or delete
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn or delete
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Gln, Gln, D-Gln or delete
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln or delete
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr, or delete
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp or delete
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln, or delete
Glycine	G	Ala, D-Ala, Pro, D-Pro, Aib, B-Ala, Acp or delete
Isolencine	I	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met, or delete
Leucine	L	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met or delete
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn or delete
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val or delete
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, Ada-A, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa or delete
Proline	P	D-Pro, L-I-thiazolidine-4-carboxylic acid, D-or L-I-oxazolidine-4-carboxylic acid (Kauer, U.S. Patent 4,511,390). or delete
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O)D,-Met(O) or delete
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(0), D-Met(0), Val, D-Val or delete.
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His or delete

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TABLE 3

IGF-I RECEPTOR COMPETITION ASSAY SUMMARY

PEPTIDE (CONC.)		PERCENT MAX. BOUND (SD)	
IGF-I (10 pM)	100		(1.1)
IGF-I (40 nM)		9.6	(0.7)
IGF-II (40 nM)		92.1	(0.7)
IGF-III (40 nM)		17.6	(2.6)
IGF-I(24-41) (100 μM)		44	(7)
IGF-I(24-41) (50 μM)		99	(6)
IGF-I(24-41) (50 μM) + IGF-II(54-67)(50 μM)		49	(11)
IGF-II(54-67)(100μM)		94	(6)
IGF-I(62-70) (100 μM)		83	(20)
IGF-I(30-41) (100 μM)		94	(1.4)
IGF-II(62-67) (100 μM)		83	(21)
IGF-II(33-40) (1mM)		92	(1.8)

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TABLE 4

PEPTIDE	PERCENT MAX. BOUND
IGF-I (4 pM)	91
IGF-I (400 pM)	30
IGF-II (200 nM)	50
IGF-II (400 nM)	23
IGF-II (33-40)(1 mM)	76
IGF-II (33-40)(.10 mM)	82
IGF-II (54-67)(.25 mM)	167
IGF-II (54-67) (.025 mM)	132

TABLE   5

ADDITIVE EFFECTS OF NGF AND IGF 1 ON CHAT ACTIVITY IN CULTURED RAT SEPTAL CELLS

Growth Factor	Concentration nM	% Increase over control
NGF	2.0	44
IGF 1	1.3	20
	12.5	50
	25.0	37
2 nM NGF + IGF 1	1.3	58
	12.5	93
	25.0	100
IGF 1		44
NGF	0.02	56
	0.2	36
	2.0	44
25 nM IGF 1 + NGF	0.02	75
	0.2	100
	2.0	94

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Other embodiments are within the following claims.
What is claimed is:

- 43 -

CLAIMS

1   1.   A method of enhancing the survival of neuronal
2 cells in a mammal, said cells being at risk of dying, said
3 method comprising administering to said mammal an effective
4 amount of at least one of the following substances:
5   IGF-I;
6   a functional derivative of IGF-I;
7   IGF-II; or
8   a functional derivative of IGF-II
9 provided that, if IGF-I or IGF-II is administered an effective
10 amount of NGF or of a functional derivative of NGF is also
11 administered.

1   2.   The method of claim 1, wherein there is
2 administered a functional derivative of IGF-I or IGF-II, and
3 said method further comprises administering an effective amount
4 of NGF or a functional derivative thereof.

1   3.   The method of claim 1, wherein said neuronal cells
2 are non-mitotic neuronal cells.

1   4.   The method of claim 1, wherein said neuronal cells
2 are cholinergic cells.

1   5.   The method of claim 1, wherein said method is used
2 for the therapeutic treatment of the deleterious effect of a
3 disease, injury or aging on said neuronal cells.

1   6.   The method of claim 5, wherein said disease is
2 Alzheimer's disease, stroke, epilepsy, amyotrophic lateral
3 sclerosis or Parkinson's disease.

1   7.   The method of claim 1, wherein at least one of a
2 fragment of IGF-I, a fragment of IGF-II, or a fragment of NGF
3 is administered to said mammal.

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1   8.   The method of claim 1, wherein said functional
2   derivative of IGF-II is IGF-II(54-67).

1   9.   The method of claim 1, wherein at least one of an
2   analog of IGF-I, an analog of a fragment of IGF-I, an analog of
3   IGF-II, an analog of a fragment of IGF-II, an analog of NGF, or
4   an analog of a fragment of NGF is administered to said mammal.

1   10. The method of claim 9, wherein one or more of
2   said analogs administered to said mammal is a polypeptide which
3   has been chemically modified in such a way as to increase its
4   ability to be transported across the blood-brain barrier.

1   11. The method of claim 10, wherein said modification
2   comprises increasing said polypeptide's lipophilicity.

1   12. The method of claim 10, wherein said modification
2   comprises glycosylation.

1   13. The method of claim 10, wherein said modification
2   comprises increasing the net positive charge on said
3   polypeptide.

1   14. The method of claim 1, wherein a combination of
2   two or more of said substances, which act additively, is
3   administered to said mammal.

1   15. The method of claim 1, wherein a combination of
2   two or more of said substances, which act synergistically, is
3   administered to said mammal.

1   16. A method of enhancing the survival of neuronal
2   cells in a mammal, said cells being at risk of dying, said
3   method comprising

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4   treating said mammal with a first treatment comprising
5   administration of a cell survival promoting amount of a growth
6   factor, or a functional derivative thereof, and then
7   treating said mammal a with second treatment
8   comprising administration of a nerve transmitter increasing
9   amount of a transmitter enhancer, or a functional derivative
10   thereof.

1   17.   The method of claim 16, wherein said second
2   treatment increases the level of a transmitter specific enzyme.

1   18.   The method of claim 16, wherein said growth
2   factor is IGF-I or IGF-II.

1   19.   The method of claim 16, wherein said functional
2   derivative of said growth factor is a functional derivative of
3   IGF-I or a functional derivative of IGF-II.

1   20.   The method of claim 16, wherein said functional
2   derivative of said growth factor comprises a fragment of said
3   growth factor.

1   21.   The method of claim 16, wherein said functional
2   derivative of said growth factors is IGF-II(54-67).

1   22.   The method of claim 16, wherein said functional
2   derivative of said growth factor comprises any of an analog of
3   said growth factor, or an analog of a fragment of said growth
4   factor.

1   23.   The method of claim 22, wherein at least one of
2   said analogs administered to said mammal is a polypeptide which
3   has been chemically modified in such a way as to increase its
4   ability to be transported across the blood-brain barrier.

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24. The method of claim 23, wherein said modification comprises increasing said polypeptide's lipophilicity.

25. The method of claim 23, wherein said modification comprises glycosylation.

26. The method of claim 23, wherein said modification comprises increasing the net positive charge on said polypeptide.

27. The method of claim 16, wherein said transmitter enhancer is NGF.

28. The method of claim 16, wherein said functional derivative of said transmitter enhancer is a functional derivative of NGF.

29. The method of claim 16, wherein said functional derivative of said transmitter enhancer comprises a fragment of said transmitter enhancer.

30. The method of claim 16, wherein said functional derivative of said transmitter enhancer comprises any of an analog of said transmitter enhancer, or an analog of a fragment of said transmitter enhancer.

31. The method of claim 30, wherein at least one of said analogs administered to said mammal is a polypeptide which has been chemically modified in such a way as to increase its ability to be transported across the blood-brain barrier.

32. The method of claim 31, wherein said modification comprises increasing said polypeptide's lipophilicity.

33.   The method of claim 31, wherein said modification comprises glycosylation.

34.   The method of claim 31, wherein said modification comprises increasing the net positive charge on said polypeptide.

35.   The method of claim 16, wherein said neuronal cells are non-mitotic neuronal cells.

36.   The method of claim 16, wherein said neuronal cells are cholinergic cells.

37.   The method of claim 16, wherein said method is used for the therapeutic treatment of the deleterious effect of a disease, injury or aging on said neuronal cells.

38.   The method of claim 37, wherein said disease is Alzheimer's disease, stroke, epilepsy, amyotrophic lateral sclerosis or Parkinson's disease.

39.   The method of claim 16, wherein said first treatment and said second treatment act additively.

40.   The method of claim 16, wherein said first treatment and said second treatment act synergistically.

41.   A method of enhancing the cholinergic activity of cholinergic neurons in a mammal, said method comprising administering to said mammal an effective amount of one or more of the following substances:

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5   IGF-I;

6   a functional derivative of IGF-I;

7   IGF-II; or

8   a functional derivative of IGF-II

9   provided that if IGF-I or IGF-II is administered NGF or a

10 functional derivative thereof is also administered.

1   42. The method claim 41 wherein there is administered

2   a functional derivative of IGF-I or IGF-II, and said method

3   further comprises administering an effective amount of NGF or a

4   functional derivative thereof.

1   43. The method of claim 41, wherein said method is

2   used for the therapeutic treatment of the deleterious effect of

3   a disease, injury, or aging on said neuronal cells.

1   44. The method of claim 42, wherein said disease is

2   Alzheimer's disease, stroke, epilepsy, amyotrophic lateral

3   sclerosis or Parkinson's disease.

1   45. The method of claim 41, wherein at least one of a

2   fragment of IGF-I, a fragment of IGF-II, or a fragment of NGF

3   is administered to said mammal.

1   46. The method of claim 41, wherein said functional

2   derivative of IGF-II is IGF-II(54-67).

1   47. The method of claim 41, wherein at least one of

2   an analog of IGF-I, an analog of a fragment of IGF-I, an analog

3   of IGF-II, an analog of a fragment of IGF-II, an analog of NGF,

4   or an analog of a fragment of NGF is administered to said

5   mammal.

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1   48. The method of claim 47, wherein at least one of
2   said analogs administered to said mammal is a polypeptide which
3   has been chemically modified in such a way as to increase its
4   ability to be transported across the blood-brain barrier.

1   49. The method of claim 48, wherein said modification
2   comprises increasing said polypeptide's lipophilicity.

1   50. The method of claim 48, wherein said modification
2   comprises glycosylation.

1   51. The method of claim 48, wherein said modification
2   comprises increasing the net positive charge on said
3   polypeptide.

1   52. The method of claim 41, wherein a combination of
2   two or more of said substances, which act additively, is
3   administered to said mammal.

1   53. The method of claim 41, wherein a combination of
2   two or more of said substances, which act synergistically, is
3   administered to said mammal.

1   54. A method of enhancing the cholinergic activity of
2   cholinergic neurons in a mammal, said method comprising
3   treating said mammal with a first treatment comprising
4   administration of a cell survival promoting amount of a growth
5   factor, or a functional derivative thereof, and then
6   treating said mammal with a second treatment
7   comprising administration of a nerve transmitter increasing
8   amount of a transmitter enhancer, or a functional derivative
9   thereof.

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55. The method of claim 54, wherein said second treatment increases the level of a transmitter specific enzyme.

56. The method of claim 54, wherein said growth factor is IGF-I or IGF-II.

57. The method of claim 54, wherein said functional derivative of said growth factor is a functional derivative of IGF-I or a functinal derivative IGF-II.

58. The method of claim 54, wherein said functional derivative of said growth factor comprises a fragment of said growth factor.

59. The method of claim 58, wherein said functional derivative of said first growth factor is IGF-II(54-67).

60. The method of claim 54, wherein said functional derivative of said growth factor comprises any of an analog of said growth factor, or an analog of a fragment of said growth factor.

61. The method of claim 60, wherein at least one of said analogs administered to said mammal is a polypeptide which has been chemically modified in such a way as to increase its ability to be transported across the blood-brain barrier.

62. The method of claim 61, wherein said modification comprises increasing said polypeptide's lipophilicity.

63. The method of claim 61, wherein said modification comprises glycosylation.

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1   64.   The method of claim 61, wherein said modification
2   comprises increasing the net positive charge on said
3   polypeptide.

1   65.   The method of claim 54, wherein said transmitter
2   enhancer is NGF.

1   66.   The method of claim 54, wherein said functional
2   derivative of said transmitter enhancer is a functional
3   derivative of NGF.

1   67.   The method of claim 54, wherein said functional
2   derivative of said transmitter enhancer comprises a fragment of
3   said transmitter enhancer.

1   68.   The method of claim 54, wherein said functional
     derivative of said transmitter enhancer comprises any of an
3   analog of said transmitter enhancer, or an analog of fragment
4   of said transmitter enhancer.

1   69.   The method of claim 68, wherein at least one of
2   said analogs administered to said mammal is a polypeptide which
3   has been chemically modified in such a way as to increase its
4   ability to be transported across the blood-brain barrier.

1   70.   The method of claim 69, wherein said modification
2   comprises increasing said polypeptide's lipophilicity.

1   71.   The method of claim 69, wherein said modification
2   comprises glycosylation.

1   72.   The method of claim 69, wherein said modification
2   comprises increasing the net positive charge on said
     polypeptide.

73.   The method of claim 54, wherein said method is
used for the therapeutic treatment of the deleterious effect of
a disease, injury, or aging on said neuronal cells.

74.   The method of claim 73, wherein said disease is
Alzheimer's disease, stroke, epilepsy, amyotrophic lateral
sclerosis or Parkinson's disease.

75.   The method of claim 54, wherein said first
treatment and said second treatment act additively.

76.   The method of claim 54, wherein said first
treatment and said second treatment act synergistically.

77.   A method of treating a head or spinal cord injury
of a mammal, or a disease condition affecting neuronal cells of
a mammal, said disease condition comprising stroke, epilepsy,
age-related neuronal loss, amyotrophic lateral sclerosis or
Parkinson's disease, said method comprising administering to
said mammal an effective amount of at least one of the
following substances:
          IGF-I;
          a functional derivative of IGF-I;
          IGF-II; or
          a functional derivative of IGF-II.

78.   The method of claim 77, wherein an additive
combination of two or more of said substances is administered
to said mammal.

79.   The method of claim 77, wherein a synergistic
combination of two or more of said substances is administered
to said mammal.

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1   80. The method of claim 77, wherein said functional
2   derivative of IGF-II is IGF-II(54-67).

1   81. The method of claim 77, further comprising
2   administering an effecitive amount of NGF or a functional
3   derivative thereof.

1   82. A method of treating a head or spinal cord injury
2   of a mammal, or a disease condition affecting neuronal cells of
3   a mammal, said disease condition comprising stroke, epilepsy,
4   age-related neuronal loss, amyotrophic lateral sclerosis or
5   Parkinson's disease, said method comprising,
6   treating said mammal with a first treatment comprising
7   administration of a cell survival promoting amount of one or
8   more of a group of substances comprising IGF-I, a functional
9   derivative of IGF-I, IGF-II, and a functional derivative of
10   IGF-II, and
11   treating said mammal with a second treatment
12   comprising administration of a nerve transmitter increasing
13   amount of a transmitter enhancer, or a functional derivative
14   thereof.

1   83. The method of claim 82, wherein said transmitter
2   enhancer is NGF, or a functional derivative thereof.

1   84. The method of claim 82, wherein the effect of the
2   administered substances is additive.

1   85. The method of claim 82, wherein the effect of the
2   administered substances is synergistic.

1   86. The method of claim 82, wherein said functional
2   derivative of IGF-II is IGF-II(54-67).

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1   87. A method of modifying a ligand capable of binding
2   to a receptor located on a cell surface, said method comprising
3   binding said ligand to a preparation of said receptor
4   prior to subjecting said ligand to a modification procedure,
5   performing said modification procedure, and
6   releasing said ligand from said receptor following
7   said modification procedure.

1   88. The method of claim 87, wherein said ligand is a
2   neuroactive polypeptide.

1   89. The method of claim 87, wherein said modification
2   comprises cationization, glycosylation, or increasing the
3   lipophilicity of the ligand.

1   90. A modified polypeptide made according to the
2   method of claim 87.

1   91. A composition of matter comprising a first
2   component from the group of purified IGF-I, a purified
3   functional derivative of IGF-I, purified IGF-II, or a purified
4   functional derivative of IGF-II, and a second component from
5   the group of purified NGF, or a purified functional derivative
6   of NGF.

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Effect of IGF-I on Spinal Cord Cholinergic Neuronal Survival
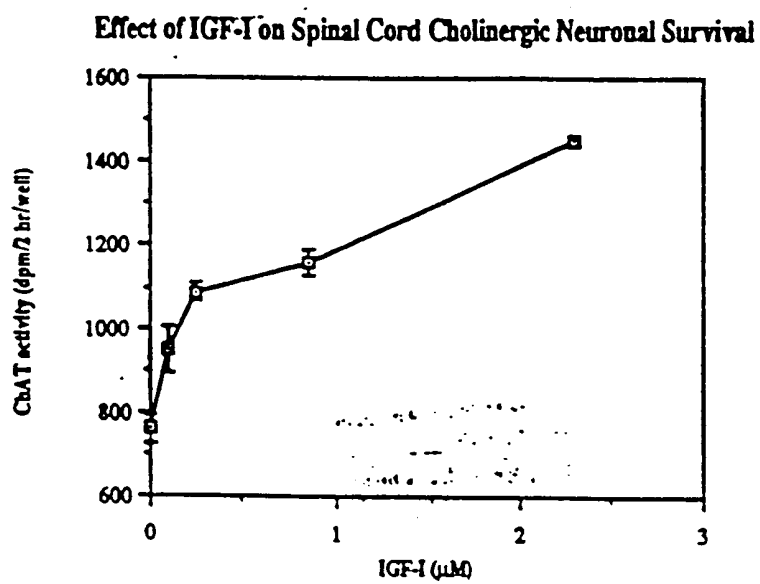
Figure 1

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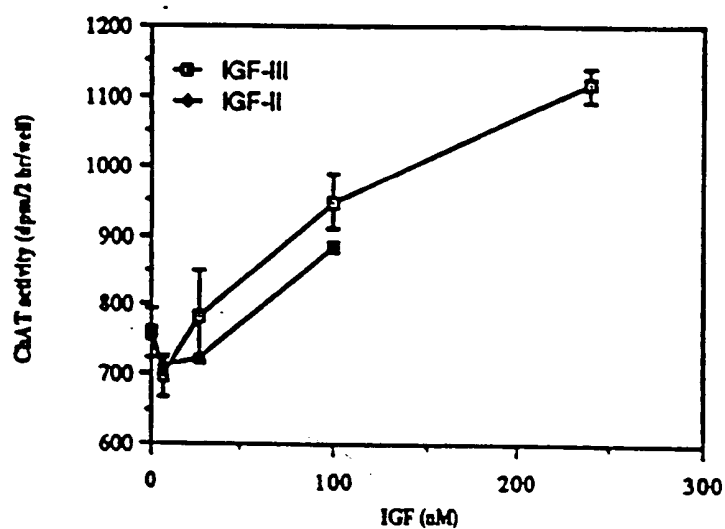Effect of IGF-II or IGF-III on Spinal Cord Cholinergic Neuronal Survival

Figure 2

$3/11$

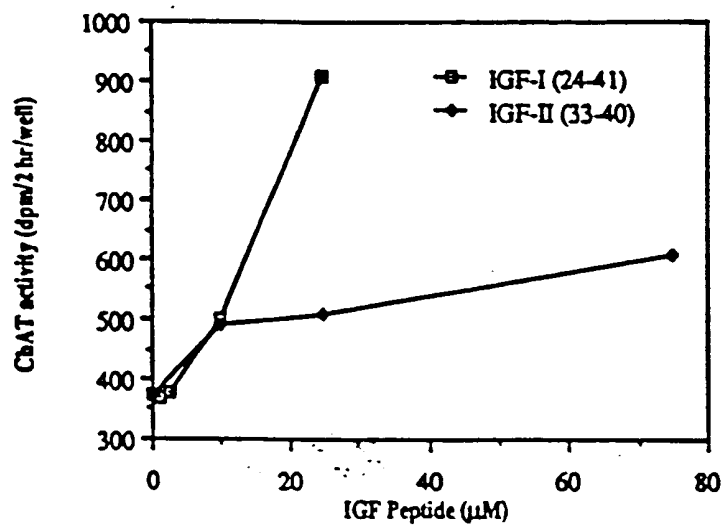Effect of IGF Fragments on Spinal Cord Cholinergic Neuronal Survival
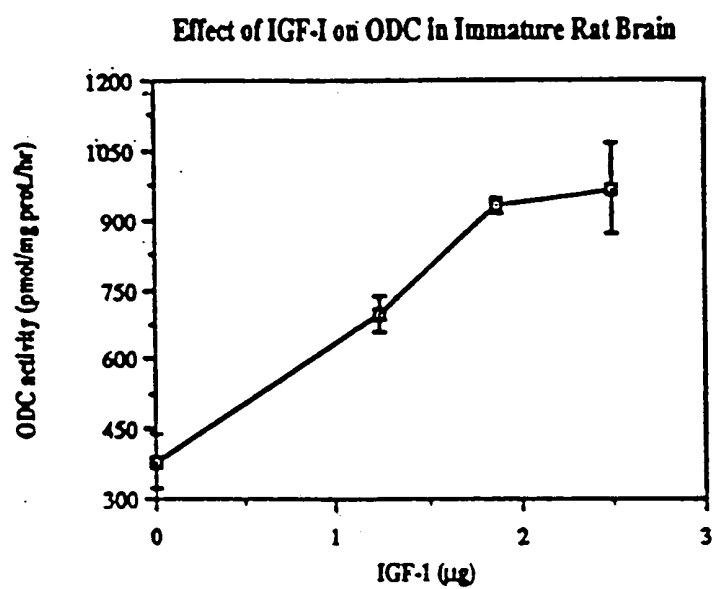

Figure 3

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Effect of IGF-I on ODC in Immature Rat Brain

Figure 4

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Effect of IGF-I or IGF fragments on ODC in Immature Rat Brain

Figure 5

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Figure 6

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Effect of {IGF-II(54-67) and IGF I on the survival of cortical cells assessed by incorporation of 3H leucine

Fig 7

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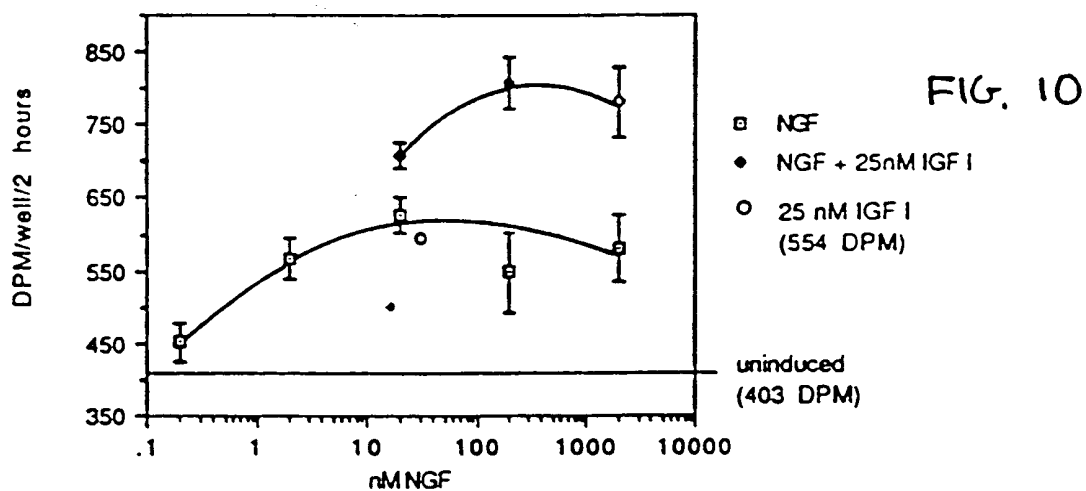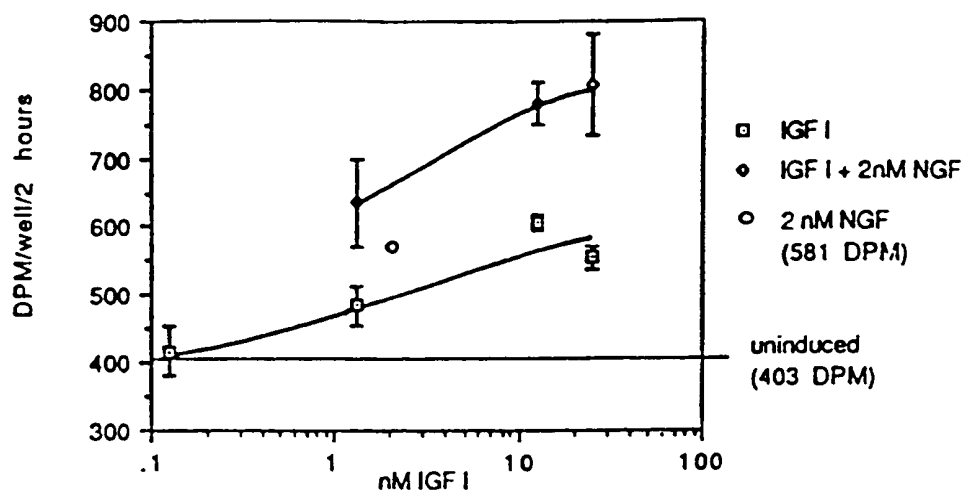Effect of *IGF-I(54-67)* and IGF I on the survival of cortical neurons assessed morphologically

Fig 8
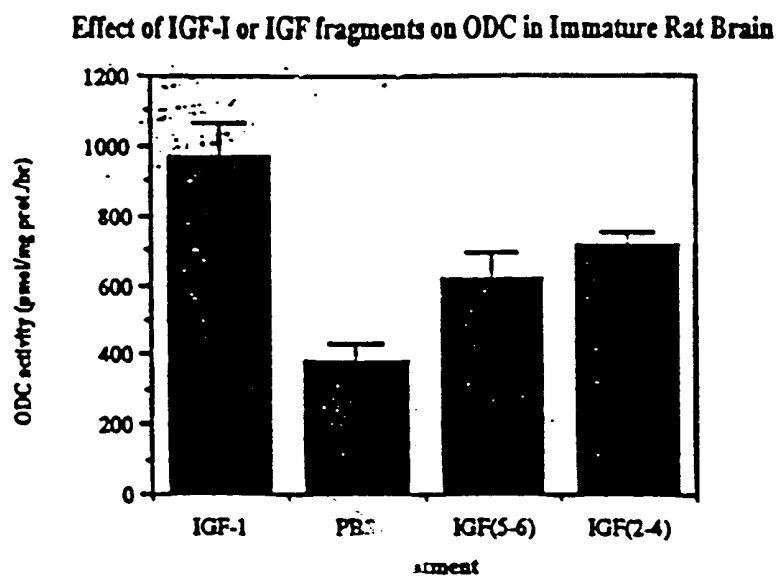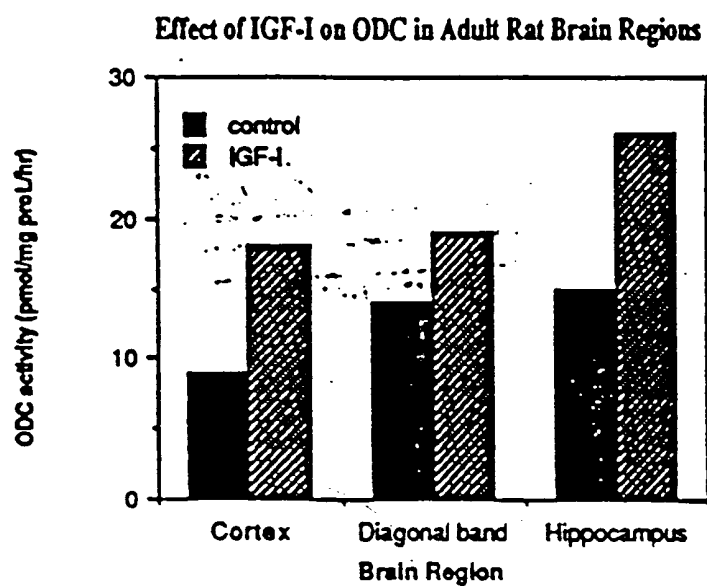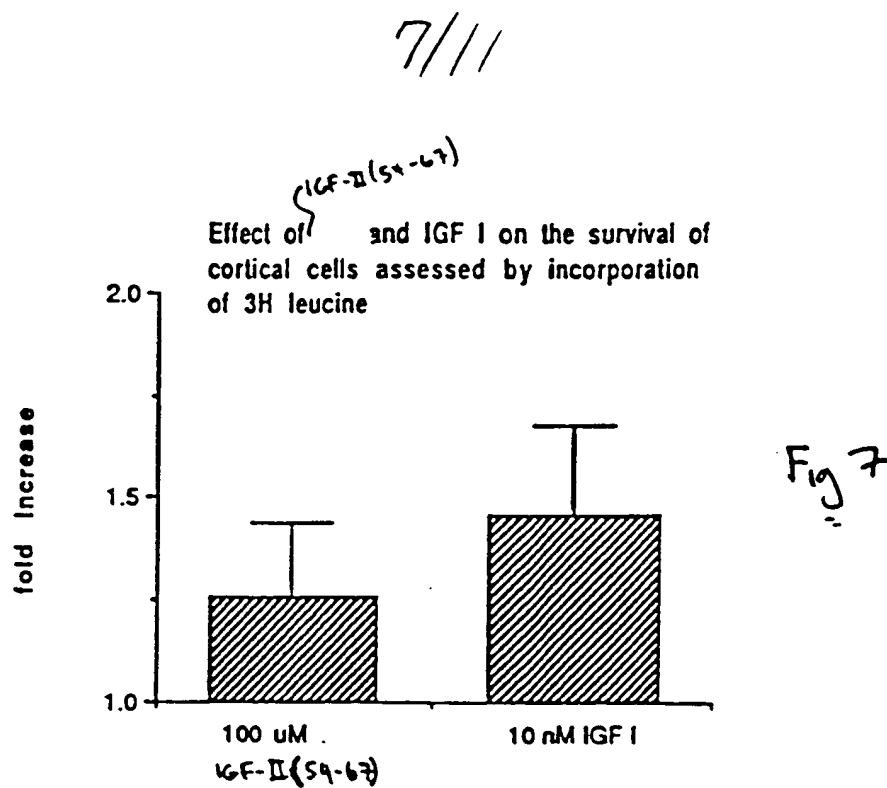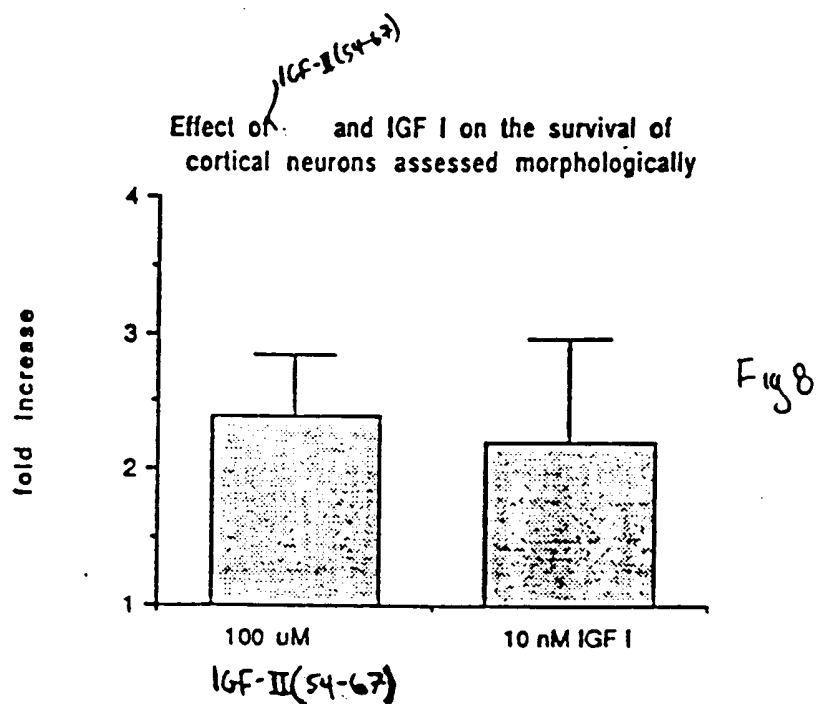
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Additive effects of NGF and IGF I on choline acetyl-
transferase activity in cultured rat septal cells

FIG. 9

□ IGF I

◊ IGF I + 2nM NGF

○ 2 nM NGF
 (581 DPM)

uninduced
(403 DPM)

FIG. 10

□ NGF

◆ NGF + 25nM IGF I

○ 25 nM IGF I
 (554 DPM)

uninduced
(403 DPM)

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Effect of Timed Additions of NGF and IGF I
on Septal ChAT Activity

A: 2 nM NGF

B: 25 nM IGF I

C: IGF I + NGF, 5 days

D: IGF I, 5 days
+ NGF added
on day 3

FIG. 11

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Effect of NGF and IGF I on the number of
ACHE positive cells in septal cultures
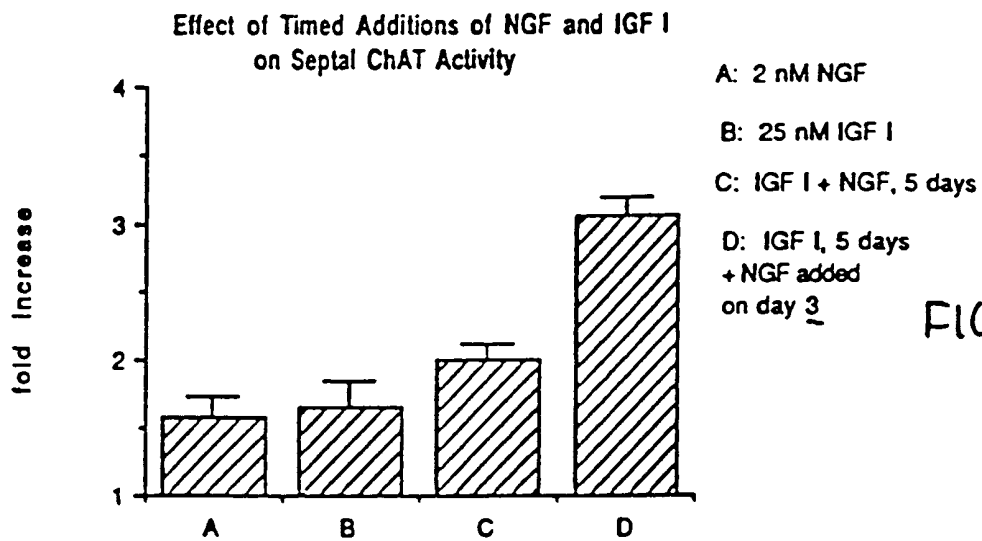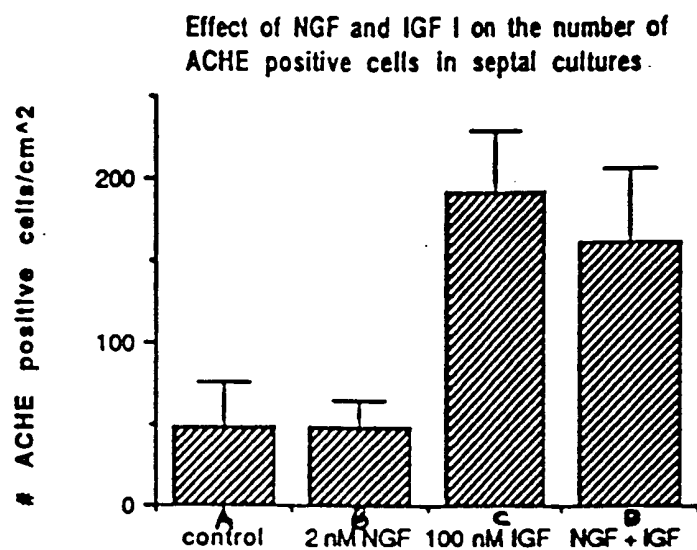
FIG. 12

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/24; A61K 37/26
US. CL.: 424/98; 514/04,12,885,903

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
US	424/98; 514/04,12,885,903

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

CAS: ON LINE AND APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	US, A, 4,341,762 (HAAST) 27 July 1982, see the entire document particularly the abstract and column 9, lines 15-27 and examples 1 and 9-12.	1-76, 81-86 and 91
Y	US, A, 4,801,575 (PARDRIDGE) 31 January 1989, abstract and column 2, lines 22-54.	87-90
X / Y	EP, A, 0,227,619 (Sara) 01 July 1987, see the entire document, especially the abstract, page 2, lines 24-48 and claims 4-5.	1,3,4 / 5-9,16,18-22, 35-38,41-47, 54-60,73,74,77, 80-83,86 and 91
Y	EP, A, 0,289,314 (Baytink) 02 November 1988, see page 3, lines 37-47.	8-10,12,19-23, 25,45-48,50, 57-60,63 and 91

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ⁸
28 August 1990	2 4 SEP 1990
International Searching Authority ¹	Signature of Authorized Officer ¹⁰
ISA/US	Fatemeh Moezie

Form PCT/ISA/210 (second sheet) (May 1986)

Category *	Citation of Document, ¹ˉ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Acta. Physiol. Scand. Vol. 126, issued 1986, H.A. HANSSON et al. "Evidence indicating trophic importance of IGF-I in regenerating peripheral nerves" see paragraph bridging pages 613 and 614.	77 82
Y	Brain Research, Vol. 485, issued 1989, J. Sjöberg "Insulin-like growth factor (IGF-1) as a stimulator of regeneration in freeze-injured rat sciatic nerve", see abstract at page 102.	77 82
A	The Merck Index, Ninth edition, published 1976 by Merck and Co., Inc. See entry no. 6306 "NGF" at page 843.	1-91
Y	WO, A, 89/01343 (PARDRIDGE et al.) 23 February 1989, see the entire document, particularly the abstract.	10,13,23,26,31, 34,48,51,61,64, 69,72